

## Water- and Air-Distributed Conidia Differ in Sterol Content and Cytoplasmic Microviscosity<sup>▽</sup>

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**Airborne and waterborne fungal spores were compared with respect to cytoplasmic viscosity and the presence of ergosterol. These parameters differed markedly between the two spore types and correlated with spore survival. This suggests that the mode of spore dispersal has a bearing on cellular composition, which is relevant for the eradication of industrially relevant fungal propagules.**

Contamination of food products by fungi often starts with dispersal vehicles that include air- and waterborne spores. The aim of this study was to assess whether air- and waterborne spores are not only different with respect to surface wettability but also have a distinct membrane and cytoplasmic composition. To this end, microviscosity and the presence of ergosterol in the plasma membrane were determined. Ergosterol is the target of many antifungals, and its presence or absence will affect sensitivity to such antifungals, including natamycin. Natamycin is considered a fungistatic antibiotic. It binds to ergosterol but is not able to disrupt the plasma membrane (9, 11). In this study, conidia of *Penicillium discolor*, *Aspergillus niger* (airborne), *Fusarium oxysporum*, and *Verticillium fungicola* (waterborne) were used. All of these species are relevant in applied situations ranging from postharvest diseases (*Aspergillus* and *Fusarium*) and food spoilage (*Penicillium*) to mycoparasitism of mushrooms (*Verticillium*). *A. niger* N402 and *P. discolor* CBS112557 were grown on malt extract agar (MEA; 7) at 25°C. *F. oxysporum* CBS116593 and *V. fungicola* MES12712 were grown on oatmeal agar (7) at 25°C. Low-temperature scanning electron microscopy of uncoated samples (8) clearly showed that the conidia of *Verticillium* and *Fusarium* were formed in large (spherical) clusters or on the surface of the colony amid the mycelium, while the other fungi showed clearly elevated spore-forming structures that formed chains of conidia (Fig. 1). Conidia of 10- to 12-day-old cultures were harvested in cold ACES buffer [10 mM *N*-(2-acetamido)-2-aminoethanesulfonic acid, 0.02% Tween 80, pH 6.8] and stored on ice before experimentation on the same day.

**Cytoplasmic microviscosity of air- and waterborne spores.** The viscosity of the interior of the cell has been correlated with the dormancy and stress resistance of fungal spores (1). Spin label electron spin resonance (ESR) spectroscopy was utilized to measure the cytoplasmic microviscosity of conidia using the spin label perdeuterated TEMPONE (4-oxo-2,2,6,6-tetramethylpiperidine-

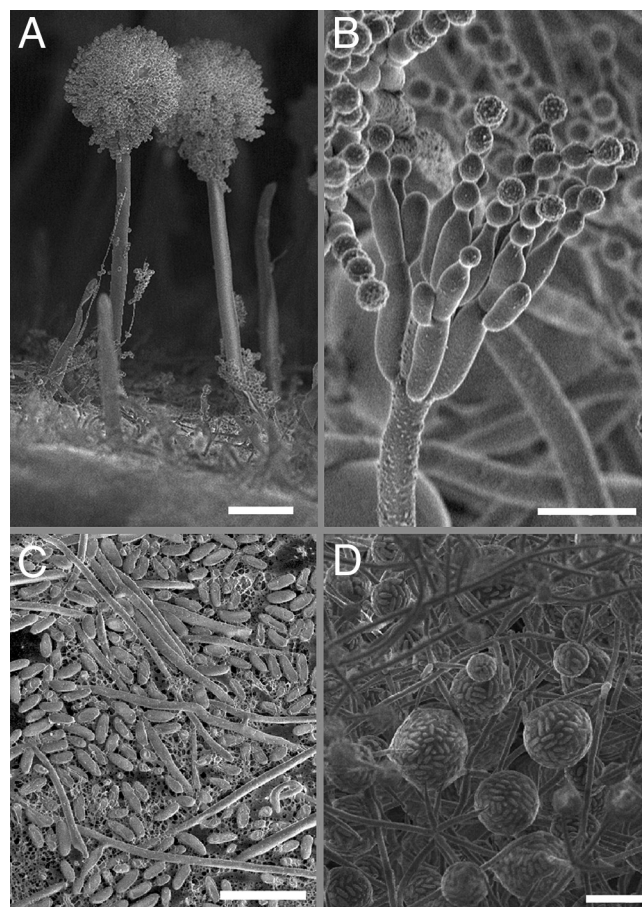


FIG. 1. Formation of conidia by *V. fungicola* (Vf), *F. oxysporum* (Fo), *P. discolor* (Pd), and *A. niger* (An) observed by scanning cryo-electron microscopy. (A) Numerous conidia of *A. niger* are formed on erect conidiophores (B) Conidia of *P. discolor* are also formed on conidiophores, and the chains of the spores are notable. (C) Conidia of *F. oxysporum* are formed within the mycelium. (D) Conidia of *V. fungicola* are formed in large clusters that coalesce to form large aggregates of spores inside the mycelium. Bars, 10  $\mu$ m.

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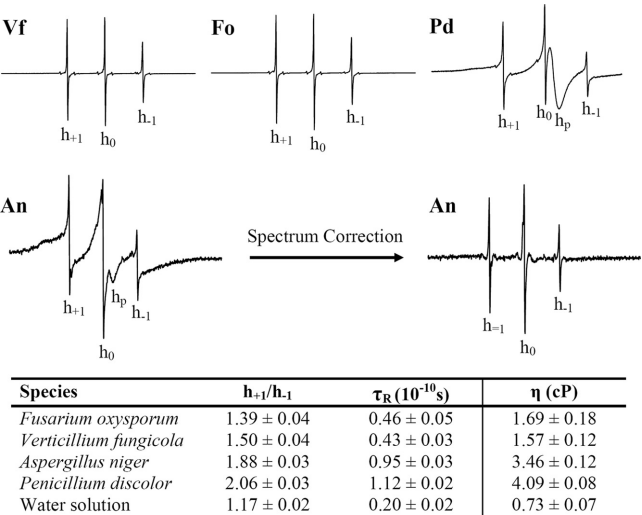


FIG. 2. ESR spectra of perdeuterated TEMPONE in dormant conidia of *V. fungicola* (Vf), *F. oxysporum* (Fo), *P. discolor* (Pd), and *A. niger* (An). The ESR spectra of TEMPONE in conidial cytoplasm contain a central line flanked by low-field and high-field lines ( $h_0$ ,  $h_{+1}$ , and  $h_{-1}$ , respectively). Spectra of *A. niger* and *P. discolor* also contain a superimposed singlet ( $h_p$ ) originating from the paramagnetic melanin present in the conidial cell wall. Because the spectrum of *A. niger* also contained a considerable proportion of a broad triplet originated from TEMPONE in a supernatant and/or cell wall, an additional spectrum correction was needed to calculate the spectral parameters of the cytoplasmic signal. The ratio of the line heights of  $h_{+1}$  and  $h_{-1}$  ( $h_{+1}/h_{-1}$ ), the rotational correlation time ( $\tau_R$ ), and the effective viscosity ( $\eta$ ) are shown in the lower panel. Data are given with the standard deviation of at least two independent experiments.

*N*-oxy; Sigma, St. Louis, MO). TEMPONE easily penetrates the cell and resides mainly in the aqueous cytoplasm. Resulting from motional averaging, the shape of ESR spectra contains dynamic information and can be used to calculate the microviscosity of an

environment where the spin label resides (2, 3, 5). ESR spectra were recorded with an X-band ESR spectrometer (EleXsys E500; Bruker Analytik, Rheinstetten, Germany). Potassium ferricyanide [ $\text{K}_3\text{Fe}(\text{CN})_6$ ] was used to broaden the signal of TEMPONE outside the spores.

The rotational correlation time of TEMPONE molecules located in the cytoplasm of fungal conidia was calculated from the ESR spectra by using the equation  $\tau_C = K\Delta W_{+1}(\sqrt{h_{+1}/h_{-1}} - 1)$  (6), where  $\Delta W_{+1}$  represents the peak-to-peak width of the low-field (left-hand) line in gauss and  $h_{+1}$  and  $h_{-1}$  are the heights of the low-field and high-field (right-hand) lines, respectively (4).  $K$  is a constant (see reference 6) with a value  $6.5 \times 10^{-10} \text{ s}$ . The rotational correlation time obtained was used to calculate cytoplasmic microviscosity by using the Stokes-Einstein relationship  $\tau_C = 4\pi(a)^3\eta/3kT$ , where  $k$  is the Boltzmann constant,  $\eta$  is the effective viscosity,  $T$  is the absolute temperature in kelvins, and  $a$  is the particle radius (2). The TEMPONE molecule is usually approximated by a rigid sphere with a radius of  $3 \text{ \AA}$  (2).

The spectra of *P. discolor* and *A. niger* possessed an additional superimposed singlet ( $h_p$ ) that was not present in conidia of *V. fungicola* and *F. oxysporum* (Fig. 2). This  $h_p$  signal originates from the melanin in the conidial cell wall (1). The singlet does not overlap with low-field and high-field peaks of TEMPONE spectra and thus allows the calculation of  $\tau_C$  with the equation. The rotation correlation times of TEMPONE in Fig. 2 indicate that TEMPONE molecules rotate more than twice as fast in the cytoplasm of water-distributed conidia as in that of air-distributed conidia. As a result, the calculated effective viscosity ( $\eta$ ) of the conidial cytoplasm in *F. oxysporum* and *V. fungicola* was  $1.69 \pm 0.18$  and  $1.57 \pm 0.12 \text{ cP}$  and for *A. niger* and *P. discolor* it was  $3.46 \pm 0.12$  and  $4.09 \pm 0.08 \text{ cP}$ , respectively (Fig. 2). For comparison, the rotational correlation time of a 1 mM solution of perdeuterated TEMPONE in

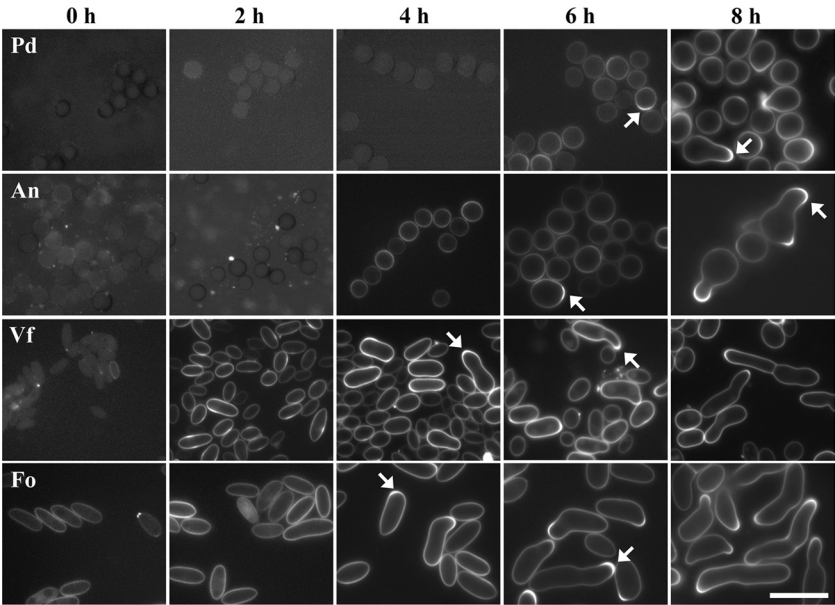


FIG. 3. Filipin staining of membrane-localized sterol in conidia of *P. discolor* (Pd), *A. niger* (An), *V. fungicola* (Vf), and *F. oxysporum* (Fo) after 0, 2, 4, 6, and 8 h of germination. *A. niger* and *P. discolor* conidia stain later than those of *V. fungicola* and *F. oxysporum* (4 to 6 h versus 0 to 2 h, respectively). Arrows point to locally increased fluorescence prior to and after the formation of germ tubes. Bar,  $10 \mu\text{m}$ .



water is  $(0.20 \pm 0.02) \times 10^{-10}$  s and the viscosity is  $0.73 \pm 0.07$  cP (Fig. 2, spectrum not shown).

**Ergosterol contents of air- and waterborne spores.** The sterol distribution of the conidial plasma membrane was assessed in freshly harvested conidia and during germination of the spores by using the fluorescent dye filipin as described previously (10). In short, spores were diluted in malt extract broth to  $10^7$ /ml. Then, 150  $\mu$ l of the suspension was placed on poly-L-lysine-coated glass coverslips and incubated at 25°C. Staining was performed with 15  $\mu$ M filipin in ACES buffer for 30 to 60 s at room temperature, followed by two washes with cold ACES buffer. Freshly harvested conidia of *V. fungicola* and *F. oxysporum* showed uniform membrane staining with filipin between 0 and 2 h of germination, while conidia of *A. niger* and *P. discolor* did not (Fig. 3). Uniform fluorescence of the plasma membrane of the latter species was only observed after 4 and 6 h of germination, respectively. At these time points, the spores were swollen. Subsequently, all of the species showed intensive staining at the presumed site of germ tube formation. Similar results were obtained when mixtures of airborne and waterborne conidia were stained, showing that small variations in the staining method were not responsible for the observed differences (data not shown). Taken together, freshly harvested waterborne conidia, but not airborne conidia, show early staining of the plasma membrane.

**Viability measurements.** Freshly harvested conidia of the fungi were incubated for 20 h in ACES buffer supplemented with 45  $\mu$ M natamycin (11). After the natamycin was removed by washing, spores were inoculated onto MEA plates and grown for 2 days. No reduction in conidial viability was observed for *A. niger* and *P. discolor* (Fig. 4B; data from three independent experiments). However, the counts of viable *V. fungicola* and *F. oxysporum* conidia were reduced to 18% and 61%, respectively. In a second approach, the fluorescent dye TOTO-1 was used as a viability marker (11). Conidia of *A. niger* and *P. discolor* did not show staining with TOTO-1. In contrast, cytoplasm and/or nuclei of *V. fungicola* and *F. oxysporum* conidia did stain, indicating a decrease in viability and membrane integrity (Fig. 4A; data from two independent experiments). The counts of viable *V. fungicola* and *F. oxysporum* conidia were reduced to 20% and 38%, respectively (Fig. 4B). Thus, some of the waterborne conidia of *V. fungicola* and *F. oxysporum* show permanent damage after treatment with natamycin, in contrast to the air-distributed conidia of *P. discolor* and *A. niger*, which did not show any staining (data not shown).

Based on low viscosity and the presence of ergosterol, we postulate that freshly harvested conidia of *F. oxysporum* and *V. fungicola* have characteristics of germinated airborne conidia. A strong decrease in cytoplasmic viscosity is also observed during the germination of stress-resistant ascospores of *Talaromyces macrosporus* (1). Furthermore, the onset of germ tube formation (Fig. 3) is faster in *F. oxysporum* and *V. fungicola*. The fact that airborne conidia are in a more dormant state helps them to survive adverse conditions in the air. This strategy also influences the efficiency of antifungals that target ergosterol. In *F. oxysporum* and *V. fungicola*, both hyphae and conidia are killed, while only germinating spores and hyphae are affected in *A. niger* and *P. discolor*.

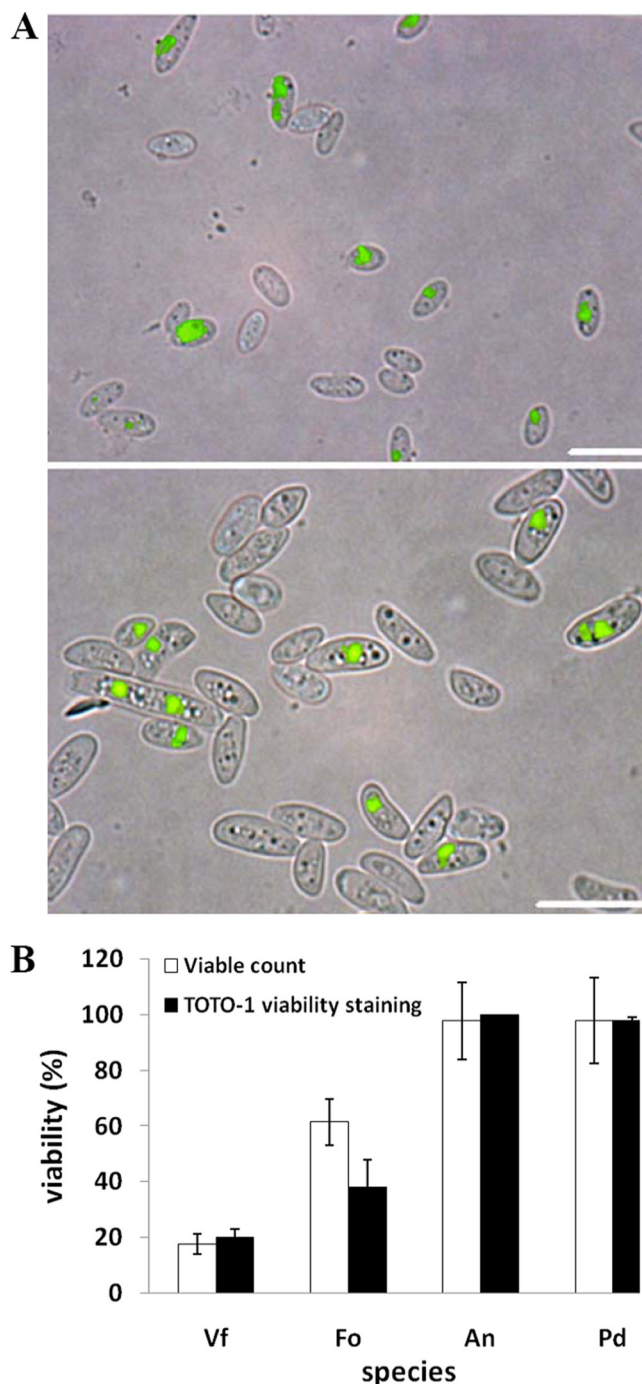


FIG. 4. Treatment of conidia of *V. fungicola* (Vf), *F. oxysporum* (Fo), *A. niger* (An), and *P. discolor* (Pd) with 45  $\mu$ M natamycin. (A) Micrographs of viability staining with TOTO-1 of *V. fungicola* (top) and *F. oxysporum* (bottom). The dye can enter the cell when the cell has lost its membrane integrity, resulting in staining of the cytosol and nucleus. Bars, 10  $\mu$ m. (B) Percentages of live cells determined by viable count measurement and after viability staining. Error bars indicate standard deviations.

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